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tRNA's Associated with the 70S RNA of Avian Myeloblastosis Virus

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The distribution of various amino acid tRNA's in the 4S RNA components of avian myeloblastosis virus (AMV) and in 4S RNA prepared from chicken embryo cells, chicken myeloblasts, and chicken livers was determined. This was done by aminoacylating the 4S RNA samples with a mixture of 17 radioactive amino acids and subsequently identifying the tRNA-accepted amino acids on an amino acid analyzer after deacylation. In embryo cells, myeloblasts, and liver, tRNA's accepting all 17 amino acids were demonstrated. "Free" AMV 4S RNA was characterized by very low quantities of glutamate, valine, and tyrosine tRNA's. RNAs accepting all 17 amino acids, with the exception of tyrosine, were shown to be present in the "70S-associated" 4S RNA which dissociates at 60 C. The bulk of the 70S-associated 4S RNA was dissociated at 60 C at low ionic strength with a concomitant conversion of 70S RNA to 35S RNA. The residual associated 4S RNA was dissociated by further heating of the 35S RNA to 80 C; tryptophan tRNA accounted for greater than 90% of the total amino acid accepting activity in this fraction. The results support other studies in suggesting that tryptophan tRNA may serve as a primer for DNA synthesis in AMV, as has been shown in Rous sarcoma virus.

Tryptophan tRNA has been identified as the major RNA primer of in vitro DNA synthesis in Rous sarcoma virus (3, 8, 9). We have shown that tryptophan tRNA is the major cellular tRNA component that hybridizes with avian myeloblastosis virus (AMV) 35S RNA in vitro (15, 16), and that the hybrid formed between AMV 35S RNA and purified tryptophan tRNA from chicken cells is a particularly efficient template-primer for DNA synthesis in vitro (16). The demonstration of an unique association, within the virus, of tryptophan tRNA and AMV 35S RNA would support the possibility that tryptophan tRNA is a biologically active primer in this virus.

Most structural studies on RNA tumor viruses have been done with either Rous sarcoma virus or AMV because of the ease with which they can be radioactively labeled and/or produced in large quantity. We have been interested in techniques for the study of viral RNA structure, particularly tRNA components, which might be applicable to a wider variety of RNA tumor viruses (15). To this end we have employed a method by which the amino acid tRNA's in the viral 4S RNA fractions can be qualitatively and semiquantitatively identified. The method depends upon the functional integrity of the tRNA's and upon the biological specificity of the aminoacyl-tRNA synthetases. Each 4S RNA fraction is aminoacylated with mixed aminoacyl-tRNA synthetases in the presence of a mixture of radioactively labeled amino acids. Unbound amino acids are removed and the tRNA-bound amino acids are deacylated and analyzed with an amino acid analyzer; from these analyses the tRNA composition of the 4S RNA sample can be inferred. A similar approach has been used by Davey and Howells to study tRNA patterns in *Drosophila melanogaster* (4). In this report we demonstrate the applicability of the method to the study of tRNA's in the 4S RNA fractions derived from an RNA tumor virus, AMV. The results are compared with those obtained using other approaches.

MATERIALS AND METHODS

Cellular tRNA preparation. Chicken embryo cells were from primary cultures grown in roller bottles in medium 199 containing 5% fetal calf serum. Myeloblasts were obtained from D. P. Bolognesi and A. J. Langois through the courtesy of J. L. Nichols, Duke University Medical Center. Livers, obtained from chickens provided by W. E. Barnett, Biology Division, Oak Ridge National Laboratory, were minced and stored at −80 C until used. Mixed tRNA was prepared from both cells and liver by methods described by Yang and Novelli (18) which included NaCl fractionation for the removal of rRNA followed by DEAE-cellulose chromatography.

These tRNA preparations were stored in 0.01 M Tris-hydrochloride, pH 7.6, 0.01 M NaCl, and 0.001 M EDTA (TNE) at −20 or −80 C.

Viral 4S RNA preparation. Chicken plasma containing AMV was obtained from J. W. Beard of Life Sciences, Inc., Gulfport, Fla. The virus was isolated and purified, and the total viral RNA was extracted as previously described (15).

Unbound or "free" viral 4S RNA was separated from 70S RNA by sedimentation through a 10 to 30% sucrose density gradient in 0.01 M Tris-hydrochloride (pH 7.6), 0.01 M NaCl, 0.001 M EDTA, and 0.05% sodium dodecyl sulfate (SDS). Centrifugation was for 5 h at 4 C in a SW41 Spinco rotor at 40,000 rpm. All RNA subunits were recovered from sucrose density gradients by ethanol precipitation and subsequent centrifugation and/or filtration through membrane filters (Millipore Corp.). Free 4S RNA was dissolved in 0.01 M Tris-hydrochloride (pH 7.6), 0.1 M NaCl, and 0.001 M EDTA and stored at −20 or −80 C.

"70S-associated" 4S RNAs were thermally dissociated under conditions of either high salt (0.01 M Tris-hydrochloride, pH 7.6, 0.1 M NaCl, 0.001 M EDTA, and 0.1% SDS) or low salt (0.02 M Tris-hydrochloride, pH 7.6, 0.01 M EDTA, and 0.1% SDS). The 70S RNA was dissolved in 0.4 to 0.5 ml of either high or low salt buffer and heated at 60 C for 3 min and quick-cooled in an ice-water bath. The 4S RNA which dissociated at 60 C was separated from 70S and/or 35S RNA by sucrose density centrifugation (7 to 10 h at 40,000 rpm). The 4S RNA regions of these gradients were pooled, and the RNA was collected and stored in TNE at −80 C.

The 70S and/or 35S RNA obtained after dissociation at 60 C (in low salt the dissociation of 70S to 35S is complete at 60 C, whereas in high salt the dissociation is only 60 to 75% complete at that temperature) was collected and dissolved in 0.4 to 0.5 ml of low salt buffer, to insure complete dissociation of both samples, and heated to 80 C for 3 min and quick-cooled. The 4S RNA which dissociates between 60 and 80 C was separated from the 35S RNA by sucrose density gradient centrifugation and stored in TNE at −80 C.

The 4S RNA samples were prepared for aminoacylation in the following manner. To insure complete removal of SDS, the samples were reprecipitated in ethanol, collected on filters, and eluted in 0.5 ml of water. The samples were lyophilized to dryness and aminoacylated directly.

Aminoacylation of tRNA. Mixed aminoacyl-tRNA synthetases were prepared from fresh chicken livers by methods already described (18). The enzymes were stored at −80 C in small aliquots in a solution containing 0.01 M KPO_4, (pH 6.5), 0.005 M $MgCl_2$, 0.25 M KCl, 0.002 M glutathione, and 15% glycerol at a protein concentration of 2.9 mg/ml. A tritium-labeled amino acid mixture was specially packaged by New England Nuclear to contain tryptophan, lysine, histidine, arginine, aspartate, threonine, serine, glutamate, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, and phenylalanine, each at a specific activity of 1,300 μCi/μmol and a concentration of 45 μM. This mixture was provided in a solution of 0.01 N HCl, and each of the amino acids except tryptophan and methionine has remained stable for at least 6 months when stored at −40 C.

Aminoacylation reaction mixtures contained 50 mM magnesium acetate, ^{3}H-labeled amino acids each at 9 μM, 0.46 mg of aminoacyl-tRNA synthetases per ml, and the indicated amounts of 4S RNA. Reactions conducted for subsequent product analysis with the amino acid analyzer were all done in 0.25 ml with the exception of the myeloblast sample which was done in 0.125 ml. In all cases, reaction mixtures were incubated at 37 C for 40 min followed by the addition of 1 ml of 0.01 M sodium acetate (pH 4.5), 0.01 M $MgCl_2$, and 0.001 M EDTA (DEAE buffer) containing 0.05 M NaCl. The samples were immediately applied to DEAE-cellulose columns (2 to 3 ml) pre-equilibrated with the same buffer at 4 C. The columns were then washed extensively with DEAE buffer containing 0.25 M NaCl to remove all amino acids not bound to tRNA. ^{3}H-labeled aminoacyl-tRNA's were then eluted with 1 M NaCl in DEAE buffer. At this point the RNA content of each sample was adjusted to an estimated 40 to 50 μg by the addition of carrier tRNA, precipitated in ethanol, and collected by membrane filtration (Millipore Corp.). Radioactive amino acids were deacylated from the tRNA by incubating the filters in 0.2 ml of 0.1 M $(NH_4)_2CO_3$ for 2 h at 37 C. The filters were then rinsed with 0.2 ml of water. Deacylation was greater than 95% complete by this method. The combined filter eluates [final 0.05 M $(NH_4)_2CO_3$] were stored at −80 C until amino acid analysis.

Amino acid analysis. Amino acid analysis was done basically as described by Moore et al. (11). The sample [0.4 ml in 0.05 M $(NH_4)_2CO_3$] was adjusted to approximately pH 2.5 by the addition of 0.6 ml of pH 2.2 diluting buffer (0.2 M sodium citrate) and applied directly to a column (0.9 by 7 cm) of Beckman PA 35 resin to separate basic amino acids. The column was eluted at 55 C with 0.2 M sodium citrate buffer, pH 5.25, at a rate of 68 ml/h. Fractions were collected directly, bypassing the ninhydrin reaction, at 1-min intervals. Acidic and neutral amino acids which eluted in fractions 2 to 10 were pooled, adjusted to pH 2.2 by the addition of 6 N HCl, and applied to a column (0.9 by 56 cm) of Beckman PA 28 resin. This column was eluted at 55 C with 0.2 M sodium citrate buffers also at a rate of 68 ml/h and 1-min fractions were collected. A buffer change from pH 3.25 to pH 4.25 was programmed at 85 min during the elution of the acidic and neutral amino acids. The radioactivity in each fraction was measured by scintillation counting of the gel obtained upon the addition of 4 ml of Aquasol (New England Nuclear). Counting was approximately 10% efficient.

RESULTS

Under the conditions used in these experiments, independent kinetic assays showed that chicken embryo cell 4S RNA accepted the 17 amino acids in the mixture to a level of 1,150 to 1,200 pmol/absorbancy unit at 260 nm. Aminoacyl-tRNA formation also was linear with the concentration of 4S RNA in the reaction. These observations indicated that a significant proportion of the tRNA within the samples was being aminoacylated under the reaction conditions used.

Cellular tRNA composition. The distribution of amino acids, which reflect the tRNA's in chicken embryo cells, is illustrated in Fig. 1A and is tabulated in Table 1. The data (Fig. 1A) show that all 17 amino acid tRNA's tested for are present in the sample, and that the synthetases for all 17 amino acid tRNA's are active. Since the amino acids used were of the same specific activity, the quantitative data (Table 1) should reflect the relative amounts of the tRNA's within the cells. Without aminoacylation data obtained with each individual amino acid, it is not possible to state positively that the distribution shown (Fig. 1A and Table 1) represents the situation in the cell. For this reason, the data are to be considered at most as being only semiquantitative. However, the percentages obtained are reproducible and are reasonable values. Independent measurements of tryptophan tRNA in chicken liver show it to be 1.5 to 2.5% of the total (9, 17), which agrees well with the data in Table 1. A notable exception would appear to be arginine tRNA. Whether the concentration of arginine tRNA in chicken cells is as high as is indicated (Table 1) or whether the specific activity of arginine in the mixture was higher than was indicated by the supplier is not certain at this time.

The reproducibility of the system and a potential pitfall in these experiments are illustrated in the second analysis, sample 2, of the chicken embryo cell 4S RNA (Table 1). Except for methionine, the results agree well with those obtained with sample 1. The apparent low level of methionine tRNA was explained by reanalysis of the ^{3}H-labeled amino acid mixture which showed the methionine content to be reduced to 3% of the original. The content of methionine sulfoxide was correspondingly increased. These results showed that the methionine in the mixture was unstable during storage at −40 C for a period of 3 months. All the other amino acids in the mixture except tryptophan were completely stable to storage under these conditons for as long as 6 months. We now routinely supplement the ^{3}H-labeled amino acid mixture with methionine and tryptophan at the time the aminoacylations are done.

The results obtained with 4S RNA from chicken livers and chicken myeloblasts are shown in Table 1. The distribution of tRNA's in these cells appears to be very similar to that seen in the embryo cells.

AMV free tRNA composition. Most of the 4S RNA isolated from AMV is not associated with the 70S RNA and is referred to as free 4S RNA. The distribution of amino acid tRNA's in AMV free 4S RNA differs markedly from that observed in chicken embryo cells or myeloblasts (Fig. 1B and Table 1). Most striking are the increases in tryptophan and proline tRNA's and the decreases in threonine and/or serine,

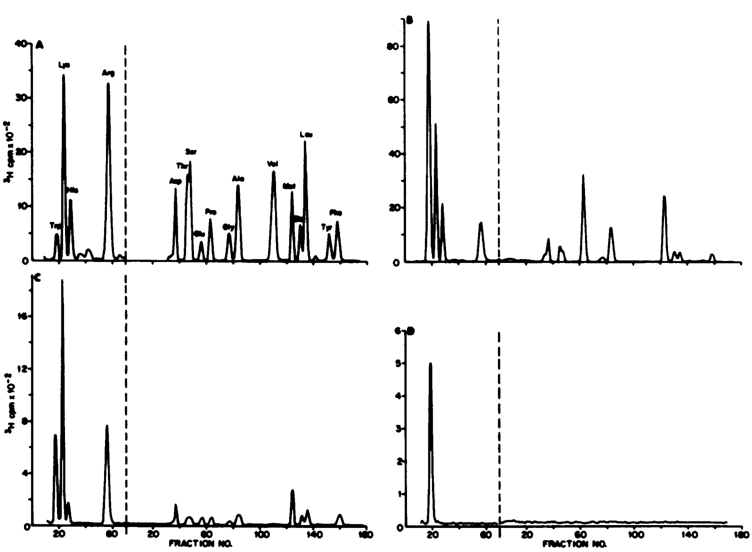

FIG. 1. *Analysis of the amino acids obtained from in vitro aminoacylated cellular and viral RNAs. (A) Chicken embryo cell 4S RNA; (B) AMV free viral 4S RNA; (C) AMV low salt 60 C viral 4S RNA; (D) AMV low salt 60 to 80 C viral 4S RNA.*

TABLE 1. *Comparison of the specific amino acid tRNA's in chicken cells and in AMV 4S RNAs*

Source of RNA	RNA (μg)	Total radio-activity[a]	Percentage of total radioactivity identified as: Trp	Lys	His	Arg	Asp	Thr and Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe
Cellular 4S RNAs																		
Chicken embryo cells (1)[b]	19	52,060	2.0	11.9	4.5	18.3	3.9	12.5	1.4	3.3	2.6	7.5	10.5	4.1	2.5	8.8	2.4	3.8
Chicken embryo cells (2)	54	150,810	2.3	11.7	4.8	18.2	4.5	13.5	1.5	3.6	3.0	8.7	10.4	0.1[c]	2.4	8.8	2.5	4.0
Chicken liver	55	88,570	2.4	10.8	4.9	15.4	5.1	14.5	1.6	5.1	2.3	8.6	11.2	0.1[c]	2.4	8.4	2.5	4.6
Myeloblasts	19	33,690	1.4	11.8	4.0	18.9	4.1	13.0	1.4	5.7	2.4	5.0	10.2	4.5	2.9	9.2	2.0	3.3
Viral 4S RNAs																		
Free 4S RNA (1)[d]	40	64,520	32.3	13.4	5.9	7.5	2.8	3.2	0.1	12.3	0.7	6.2	0.1	10.6	1.7	1.4	0.3	1.4
Free 4S RNA (2)[e]	51	83,830	30.9	13.4	5.5	6.8	3.1	3.6	0.1	13.8	0.8	7.1	ND[f]	9.2	2.1	1.7	0.3	1.6
Free 4S RNA (3)[d]	40	52,950	27.6	14.0	7.4	7.9	3.7	3.9	ND	19.6	1.1	6.8	ND	1.8[c]	1.6	2.0	0.6	2.0
Free 4S RNA (4)[d]	43	55,920	27.0	16.6	7.4	8.3	3.4	4.4	ND	16.9	0.9	7.2	ND	1.7[c]	1.7	1.9	0.6	2.0
70S-associated 4S RNA																		
High salt 60 C	–[g]	7,950	20.4	20.3	4.1	25.2	3.7	3.0	1.4	1.9	1.1	4.8	0.4	6.6	1.6	2.7	ND	2.9
High salt 60 to 80 C	–	1,010	54.5	35.6	ND	4.3	ND	1.4	1.4	ND	ND	1.4	ND	ND	ND	1.5	ND	ND
Low salt 60 C (1)	–	10,140	16.4	31.9	2.9	21.5	2.9	2.7	1.6	1.7	1.0	3.2	0.6	5.9	1.8	2.8	ND	2.9
Low salt 60 C (2)[h]	–	6,450	29.1	29.7	2.8	16.8	2.3	2.0	1.2	3.5	0.6	3.0	0.5	3.7	1.0	1.9	ND	1.8
Low salt 60 to 80 C (1)	–	1,210	98.6	1.4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Low salt 60 to 80 C (2)[h]	–	890	85.0	9.2	ND	5.8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

[a] Sum of radioactivities eluted from the amino acid analyzer in peak positions corresponding to the indicated amino acids.

[b] Numbers in parentheses indicate different RNA samples.

[c] Nonsaturating concentrations of methionine in aminoacylation reaction.

[d] Samples isolated from three different lots of plasma.

[e] Same as free 4S RNA (1) except for purification by DEAE-cellulose chromatography.

[f] ND, None detected.

[g] The high salt 60 C and 60 to 80 C samples and the low salt 60 C and 60 to 80 C samples (1) were derived from equivalent portions of 70S RNA (190 μg each). The low salt samples (2) were derived from 140 μg of 70S RNA.

[h] These samples (2) were prepared from a different lot of plasma than samples (1).

glutamate, glycine, valine, leucine, and tyrosine tRNA's. The specific amino acid acceptor activity of AMV free 4S RNA is about half that observed with chicken embryo cell 4S RNA (Table 1). No attempt was made in these studies to isolate pure 4S RNA molecules, and therefore the free 4S RNA samples may contain molecules as large as 10S. This could account for the lowered specific activity. To determine whether there might be contaminants capable of specifically inhibiting glutamate and valine tRNA synthetases, free 4S RNA was further purified by DEAE-cellulose chromatography as was used in the preparation of cellular 4S RNA. Free 4S RNA treated in this manner had an amino acid tRNA distribution pattern indistinguishable from the untreated sample (compare free 4S RNA samples 1 and 2, Table 1). The same trend regarding tryptophan, proline, glutamate, and valine tRNA's was observed with AMV free 4S RNA samples obtained from two other virus lots (free 4S RNA samples 3 and 4, Table 1).

AMV 70S-associated tRNA composition. In addition to tRNA in the free 4S RNA of the virus, there is also tRNA in the 70S-associated 4S RNA of the virus (7, 12). Using high salt conditions for thermal dissociation, the in vitro template activity of AMV 70S RNA is lost with a T_m of 74 C, whereas under conditions of low salt the T_m is shifted downward to 68 C (unpublished data).

At 60 C, under either condition of salt concentration, approximately 90% of the tRNA is removed from the 70S RNA (Table 1). With the exception of tyrosine tRNA, all 17 amino acid tRNA's were found in the 4S RNA fractions which were dissociated from 70S RNA at 60 C (Fig. 1C and Table 1). As was the case for the free 4S RNA samples, the basic amino acid tRNA's, tryptophan, lysine, and arginine, are predominant in these samples (Table 1). Our studies have shown that under low salt conditions of dissociation, the 70S RNA is completely converted to 35S RNA at 60 C, yet greater than 75% of the template-primer activity remains. That the 70S RNA molecule per se is not required for template-primer activity was first shown in RSV by Canaani and Duesberg (1). Further heating of the 35S RNA to 80 C abolishes greater than 80% of the template-primer activity measured in vitro (unpublished data). Greater than 90% of the tRNA in the 4S RNA fraction which dissociates between 60 and 80 C is tryptophan tRNA (Fig. 1D and Table 1). Detection of residual amino acid tRNA's, e.g., lysine and arginine tRNA's, in the 60 to 80 C 4S RNA sample obtained under dissociation conditions of high salt, is consistent with the observation that at 60 C the 70S to 35S conversion is only 60 to 75% completed.

DISCUSSION

The specificity of the tRNA aminoacylation reaction has been used to determine the distribution of the various amino acid tRNA's within the different 4S RNA fractions obtained from AMV. Active synthetase activity for all 17 amino acid tRNA's was demonstrated. Comparison of the tRNA distribution within the 4S RNA samples obtained from chicken liver, chicken embryo cells, and chicken myeloblasts showed the three samples to be very similar. Significant differences between the tRNA distribution in these cells and, for example, mouse fibroblasts, are demonstrable by these techniques (unpublished data).

That the tRNA in AMV free 4S RNA is not due to random packaging of the total cellular tRNA as the virus buds off the cell membrane is indicated by these experiments. Relative to myeloblasts, AMV free 4S RNA contains higher levels of tryptophan and proline tRNA's and lower levels of threonine and/or serine, glutamate, glycine, valine, leucine, and tyrosine tRNA's. These differences are in excellent agreement with the results obtained by Travnicek (13). Carnegie et al. (2) also concluded that the valine tRNA content of AMV free 4S RNA was either very low or absent. The discrepancy between these results and those of Erikson and Erikson (6) which showed the valine tRNA content of AMV free 4S RNA to be equal to or greater than that found in myeloblasts is immediately apparent. The reproducibility of our experiments and the failure to remove a hypothetical specific glutamate or valine tRNA synthetase inhibitor by DEAE-cellulose treatment of the free 4S RNA sample make it unlikely that glutamate or valine tRNA's are present but are not aminoacylated. None of these workers (2, 6, 13) tested their viral 4S RNA preparations for tryptophan tRNA. For that matter, it should be pointed out that in this study we can make no claims regarding cysteine, glutamine, and asparagine as they were not included in our amino acid mixture. Our experiments clearly support the previous contentions (2, 6, 13) that AMV free 4S RNA does not contain tRNA's in the same proportions as does the total 4S RNA of the cell of origin. Perhaps these results reflect tRNA compartmentalization within the cell and are indicative of the tRNA distribution near the membrane of the cell.

Approximately 90% of the tRNA associated with AMV 70S RNA can be dissociated by heating to 60 C. Of the 17 amino acid tRNA's tested for in this fraction, all were present except tyrosine tRNA. The basic amino acid tRNA's, tryptophan, lysine, and arginine, are particularly prominent in the 4S RNA which dissociates from 70S RNA at 60 C. It is of interest that glutamate and valine tRNA's which are notably low or absent in the free 4S RNA are present in detectable quantity in the 60 C 4S RNA fractions.

In other studies we have shown that little of the template-primer activity of 70S RNA is lost by heating at 60 C (unpublished results). Therefore, the primer molecules should be present in the 60 to 80 C 4S RNA fraction. Of the identified tRNA's in this fraction, approximately 90% are tryptophan tRNA. If a tRNA is indeed the primer for DNA synthesis in AMV, it would appear to be tryptophan tRNA. The tenacity of tryptophan tRNA and lysine tRNA binding to viral 35S and 70S RNAs is consistent with our previous results obtained by in vitro hybridization of cellular tRNA with AMV 35S RNA (16). Purified tryptophan tRNA but not partially purified lysine tRNA, when hybridized to AMV 35S RNA, has been shown to be an effective primer of reverse transcription in vitro (16). However, the present study suggests that the conversion of 70S RNA to 35S RNA might be closely coordinated with the removal of lysine tRNA as is indicated by the results obtained with the 70S-associated 4S RNAs derived under conditions of high salt. Experiments are being conducted to investigate the possibility that lysine tRNA is an important structural component of AMV 70S RNA.

Calculations made from the data presented in Table 1 show that tryptophan, lysine, and arginine tRNA's are present in the total 70S-associated 4S RNA at a level of one to two molecules per 70S RNA molecule. This estimate for tryptophan tRNA is similar to that calculated from our in vitro hybridization experiments (16). The next most abundant tRNA, methionine tRNA, is present at levels less than one molecule per four 70S RNA molecules. Since lysine, arginine, and methionine tRNA's are all completely dissociated at 60 C in low salt, whereas template-primer activity is reduced less than 25%, it is considered unlikely that they comprise a significant fraction of the primer activity as measured in vitro. These results, together with our inability to produce an effective template-primer by hybridization of methionine tRNA with AMV 35S RNA (16), do not support the suggestion of Elder and Smith (5) that methionine tRNA might serve as a primer in AMV.

These results show that in AMV, as has been shown in Rous sarcoma virus (3, 8, 9), tryptophan tRNA is the tRNA most intimately associated with the viral 70S RNA. Our previous work with AMV (16) has shown that tryptophan tRNA can be an effective primer of DNA synthesis in vitro. Whether tryptophan tRNA is a primer for reverse transcription of AMV 70S RNA in vivo remains to be seen. A recent report has indicated that the in vivo primer RNA used in Rous sarcoma virus replication may be larger than 4S (10).

We have extended the use of the technique described in this paper to the study of 70S-associated tRNA's in murine RNA tumor viruses. In the AKR murine leukemia virus (14) and in Rauscher leukemia virus, proline tRNA appears most tightly associated with the 35 to 70S RNA, suggesting that there may be species specificity with regard to potential RNA primers in RNA tumor viruses.

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